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(54) Title: HAIR FOLLICLE DNA DELIVERY SYSTEM

(57) Abstract

A novel method and delivery system for delivering nucleic acids to a mammalian cell *in vivo* by topical application of the nucleic acid and the delivery system has been developed. The method and delivery system utilize a liposomal composition having non–ionic lipids and a cationic lipid. The method and delivery system are particularly well suited for gene therapy of dermatological disorders including neoplastic disease and alopecia by topical administration.

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HAIR FOLLICLE DNA DELIVERY SYSTEM

Background of the Invention

. Topical deliver- of gene vectors to cells within the skin is an attractive strategy for gene therapy of many human diseases, including a number of dermatological conditions thought to be mediated by abnormal regulation of soluble cytokines (Kondo et al. (1995) *J Invest Dermatol* 105:334-338; Hammerberg et al. (1992) *J Clin Invest* 90:571-583; Debets et al. (1995) *J Invest Dermatol* 105:480; Hoffmann et al. (1995) *J Invest Dermatol* 105:467; Wenzel et al. (1995) *I Invest Dermatol* 105:467). However, initial attempts at gene delivery to living skin have been hampered by problems associated with the delivery of charged macromolecules beyond the stratum corneum. Thus, these methods have focused mainly on parenteral methods. Such methods have included direct injection of plasmid DNA into the upper epidermis as well as pneumatic acceleration of metallic microparticles coated with plasmid DNA into the skin (Hengge et al. (1995) *J Invest Dermatol* 105:448; Alexander et al. (1994) *Gene Ther.* 1: Suppl. 1:S57). Although some degree of skin cell transfection has been achieved by these methods, neither appears likely to allow for the delivery of plasmid DNA to a large number of target cells on a cost effective therapeutic basis.

Although recent studies have reported promising initial results using cationic liposomes for topical transfection, questions remain regarding the feasibility of these methods for the delivery of potentially therapeutic transgenes (Li L. and Hoffman R.M. (1995) *Nat Med* 1:705-706; Alexander M.Y. and Akhurst R.J. (1995) *Hum Mol Genet* 4:2279-2285). Further, the effects of composition and preparation methods of the liposomal formulations on the integrity and stability of expression plasmid DNA have not been addressed.

The successful treatment of cutaneous diseases with transgenes relies on an ability to effectively deliver them to appropriate sites within the skin. So far it has proven almost impossible to control many of these skin disorders using conventional dermatological formulations. Accordingly, the development of pharmaceutical reagents that can mediate transfection of epidermal cells would have far reaching experimental and therapeutic applications. For topical gene therapy to be successful, it is necessary to optimize delivery of recombinant DNA to accessible target cells within living skin strata using vehicles that can overcome the formidable permeability barriers of the skin and its appendages.

Accordingly, an object of the invention is to provide a method for delivering a nucleic acid to a mammalian skin cell *in vivo* by topical admininstration of the nucleic acid and a liposomal delivery system to a portion of skin having hair follicles.

A further object of the invention is to provide a liposomal delivery system, containing a combination of non-ionic and cationic lipids, for delivering a nucleic acid to a perifollicular cell *in vivo*.

Another object of the invention is to provide a method for treating a skin disorder by gene therapy using topical administration of a nucleic acid and a liposomal delivery system to a skin having hair follicles.

These and other objects and features of the invention will be apparent from the following description and the drawing.

Summary of the Invention

The invention pertains to a method of delivering a nucleic acid to a mammalian cell *in vivo*. The method comprises providing a liposomal delivery system for the nucleic acid, wherein the liposomal delivery sytem contains a lipid vesicle and a cationic lipid. The preferred lipid vesicle has a primary wall material and a secondary wall material, wherein the primary wall material is preferably selected from the group consisting of C_{12}-C_{18} glyceryl mono- and diesters and the secondary wall material is preferably selected from the group consisting of quaternary dimethyldiacyl amines, polyoxyethylene acyl alcohols, polygycerol fatty acids, and sorbitan fatty acid esters. The method further includes incorporating the nucleic acid into the liposomal delivery system to produce a nucleic acid/liposomal delivery system and topically administering the nucleic acid/liposomal delivery sytem to the mammalian cell at a location of the skin having hair follicles.

The invention is based at least in part on the discovery that a non-ionic liposomal formulation containing a non-ionic liposome such as one which form glyceryl dilaurate, cholesterol, and polyoxyethylene-10-stearyl ether, and a cationic lipid such as 1,2-dioleoyloxy-3(trimethylammonio)propane could efficiently mediate transfection of a nucleic acid into perifollicular cells *in vivo* following topical administration. Accordingly, the invention provides a method wherein a nucleic acid is delivered to cells which are in proximity of a hair follicle and the nucleic acid is introduced in these cells via topical administration of a non-ionic/cationic liposomal delivery system and the nucleic acid.

In a preferred embodiment, the mammalian cell is associated with follicular units. The mammalian cell can be associated with a pilosebaceous unit. Alternatively, the mammalian cell can be a follicular cell, e.g., a perifollicular cell.

The invention further provides a nucleic acid/liposomal delivery system for delivering a nucleic acid topically to a mammal *in vivo*. The nucleic acid/liposomal delivery system contains a nucleic acid and a liposomal delivery system for the nucleic acid. The liposomal delivery sytem includes a lipid vesicle with a primary wall material, and a secondary wall material, and a cationic lipid. The primary wall material is preferably selected from the group consisting of C_{12}-C_{18} glyceryl mono-and diesters and the secondary wall material is preferably selected from the group consisting of quaternary dimethyldiacyl amines, polyoxyethylene acyl alcohols, polyglycerol fatty acids, and sorbitan fatty acid esters. The preferred cationic lipid is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium bromide (DOTMA), 1,2-dimyristyloxypropyl-N,N-dimethyl-hydroxyethyl ammonium bromide (DMRIE), [N-(N', N'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), dioctadecylamidoglycyl spermidine (DOGS), dimethyl dioctadecylammonium bromide (DDAB), dioleoyl phosphatidylethanolamine (DOPE), 2,3-dioleoyloxyl-N-[2(sperminecarbozamide-0-ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1-[2-(oleoyloxy)-ethyl]-2-oleyl-3-(2hydroxyethyl) imidazolinium chloride (DOTIM), 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP), 1,2-diacyl-3-trimethylammonium propane (TAP), 1,2-diacyl-3-dimethylammonium propane (DAP), and fatty acid salts of quaternary amines. TAP and DAP can have various chains (14:0, 16:0, 18:0, and 18:1) and various acyl groups (dimyristoyl, dipalmitoyl, distearoyl, and dioleoyl). In a much preferred embodiment, the cationic lipid is DOTAP.

More preferably, the primary wall material of the lipid vesicle is selected from the group consisting of glyceryl dilaurate, glyceryl distearate, and their mixtures. A more preferred secondary wall material of the lipid vesicle is polyoxyethylene-10-stearyl ether.

The nucleic acid to be delivered to a mammalian cell can contain a transgene. In a preferred embodiment of the invention, the transgene encodes a protein. The protein can be a ligand, a receptor, an agonist of a ligand, an agonist of a receptor, an antagonist of a ligand, or an antagonist of a receptor. For example, the protein encoded by the transgene can be an IL-1 receptor antagonist. Alternatively, the protein can be a transdominant negative hormone receptor, such as an androgen receptor. In yet another embodiment, the nucleic acid expresses an antisense RNA or a ribozyme.

The method of the invention and the nucleic acid/liposomal delivery system of the invention can be used to treat various conditions, such as dermatological disorders. Accordingly, the invention pertains to methods of treatment of disorders selected from the group consisting of infectious diseases, neoplastic diseases, autoimmune disorders,

alopecia, aquired conditions, acne, baldness, atopic dermatitis, and eczema. The autoimmune disorder can be psoriasis or alopecia areata.

Further within the scope of the invention are kits comprising the liposomal delivery system and instructions for use. In one embodiment, the invention provides a kit containing some or all of the materials for forming the liposomal delivery system packaged separately and instructions explaining the preparation of the delivery system. In yet other embodiments, the kits can further comprise a nucleic acid, either together with the liposomal delivery system, or in a separate container.

Brief Description of the Drawings

Figure 1 shows a Southern blot containing DNA from hamster ear treated with a single dose of pSG5IL-1ra and sacrified 4 hours (4), 6 hours (6), 8 hours (8), 12 hours (12) and 24 hours (24) later, or treated with buffer alone (C1) or with aqueuos DNA (C2), hybridized with a probe specific for human IL-1ra.

Figure 2 (Panels A-C) are graphs reprenting the amount of IL-1ra present in ventral skin (Panel A), auricular carticlage (Panel B), and dorsal skin (Panel C) of hamster ear from 1 to 8 days following twice daily topical application of NC liposomes with or without pSG5IL-1ra DNA for three days to the ventral surface of the hamster ear.

Figure 3 is a graph showing the amount of IL-1ra present in the ventral surface of hamster ear 24 hours following twice daily topical application of NC liposomes (NC), NC liposomes with pSG5IL-1ra DNA (NC + DNA), phospholipidic/cationic liposomes (PC), phospholipidic/cationic liposomes with pSG5IL-1ra DNA (PC + DNA), or pSG5IL-1ra DNA alone, for three days to the ventral surface of the hamster ear.

The following description will more fully elucidate the invention.

Detailed Description of the Invention

The invention pertains to a method for delivering a nucleic acid to a mammalian cell. In a preferred embodiment of the invention, the cell is *in vivo*. The method comprises providing a liposomal delivery system for the nucleic acid, the liposomal delivery system comprising a lipid vesicle having a primary wall material and a secondary wall material, and a cationic lipid. The method further comprises incorporating the nucleic acid into the liposomal delivery system to produce a nucleic

acid/liposomal delivery system and topically administering the nucleic acid/liposomal delivery system to the mamallian cell. In a preferred embodiment, the mamallian cell is at a location of the skin having hair follicles. The primary wall material is preferably selected from the group consisting of C_{12}-C_{18} glyceryl mono- and diesters. The secondary cell wall material is preferably selected from the group consisting of quaternary dimethyldiacyl amines, polyoxyethylene acyl alcohols, polyglycerol fattly acids, and sorbitan fatty acid esters.

The invention further pertains to a nucleic acid/liposomal delivery system for delivering a nucleic acid topically to a mammal *in vivo*. The nucleic acid/liposomal delivery system comprises a nucleic acid and a liposomal delivery system for said nucleic acid. The liposomal delivery sytem comprises a lipid vesicle having a primary wall material, a secondary wall material, and a cationic lipid. The primary wall material is preferably selected from the group consisting of C_{12}-C_{18} glyceryl mono- and diesters. The secondary wall material is preferably selected from the group consisting of quaternary dimethyldiacyl amines, polyoxyethylene acyl alcohols, polygycerol fatty acids, and sorbitan fatty acid esters.

The term "liposomal delivery system" which is used interchangeably herein with the term "topical gene delivery system" is intended to include a system, which mediates or facilitates introduction of a macromolecule, e.g., a nucleic acid, into a cell using liposomes, lipid vesicles, or other lipid compositions. The term "nucleic acid/liposomal delivery system" is intended to include a complex comprising the liposomal liposomal delivery system and a nucleic acid. The term "non-ionic/cationic liposomal delivery system" is used interchangeably herein with the terms "NC liposomal delivery system", "NC liposomal suspension", and "NC liposomal formulation", and is intended to include a liposomal delivery system having non-ionic lipid vesicles and cationic lipids.

The term "delivering a nucleic acid to a cell" is used interchangeably herein with the term "transfecting a cell with a nucleic acid" and "introducing a nucleic acid into a cell". Accordingly, the invention pertains to a method for transfecting a mammalian cell with a nucleic acid.

The term "incorporating a nucleic acid into a liposomal delivery system" is intended to include adding a nucleic acid to a liposomal delivery system, such that the nucleic acid/liposomal delivery system formed is capable of delivering the nucleic acid to a cell *in vivo*.

In a preferred embodiment of the invention, the mammalian cell is a cell from a skin having hair follicles. The skin containes several layers, including the epidermis, which is the outside layer of the skin, the dermis, and the subcutaneous layer. The

epidermis includes at least the following cell layers: the stratum corneum, which is the outside layer of the skin, the stratum granulosum, the stratum spinosum, and the stratum basale (germinativum). The dermis is defined as the region between the epidermis and the subcutaneous layer and includes, but is not limited to, the papillary region or layer, the reticular region or layer, hair follicle roots, sensory nerves, sudoriferous glands and ducts, and sebaceous glands. The subcutaneous layer includes, but is not limited to, arteries, autonomic motor nerves, veins, sudoriferous glands, and lamellated (Pacinian) corpuscules.

Hair follicles can be thought of as a self contained organ system that bridges dermis and epidermis. At the epidermal surface the follicular opening ranges from 15-100 microns, while along the depth of the hair shaft the physical distance between hair shaft and perifollicular cells ranges from 3-10 microns. The term "perifollicular cell" is intended to include a cell, e.g., follicular cell, located in close proximity to the hair shaft. Close proximity is intended to include within several cell layers. A perifollicular cell can be a cell lining the perifollicular space, or a cell further away from the perifollicular space. The term "perifollicular space" is intended to include the space between the hair shaft and the cells, e.g., perifollicular cells, lining the hair shaft. A hair follicle is connected to pilosebaceous (oil) glands through pilosebaceous ducts. The term "pilosebaceous unit" is intended to include pilosebaceous glands and ducts. The ducts of the pilosebaceous glands open directly into this perifollicular space. Thus, the anatomical structure of the follicle is well suited as a receptacle for introduction into skin of exogenous liquids and microscopic particulates, such as non-ionic/cationic liposomal formulations of the invention and their contents. Accordingly, the invention provides methods for introducing a nucleic acid into cells proximate to the perifollicular space or ducts connected to the perifollicular cell, such as pilosebaceous ducts. Thus, the invention provides methods for transfecting perifollicular cells, e.g., perifollicular fibroblasts along the interior length and base of the hair shaft. The invention further provides methods for transfecting sebocytes (gland lining cells), such as sebocytes present within pilosebaceous glands, and keratinocytes, such as keratinocytes located near the opening of the follicle. Other target cells include cells from any of the layers of the epidermis and the dermis which are close to a hair shaft.

The root of a hair follicle, also termed hair root, includes the medulla, the cortex, and the cuticle of the hair. On the outside of the cuticle is the internal root sheath, followed by the external root sheath and the connective tissue sheath, containing blood vessels. Accordingly, the invention also provides methods for delivering nucleic acids to cells from these layers or tissues.

Non-ionic/cationic lipid formulations

The follicular openings occupy only about 0.1% of the total skin surface. Nevertheless, a number of studies have clearly demonstrated that the follicular pathway is important for a surprisingly wide range of compounds (Scheuplein (1967) *J Invest Dermatol* 48:79-88; Behl et al. (1981) *J Pharm Sci* 70:835-837; Illel et al. (1991) *J Pharm Sci* 80:424-427). Liposomally aided follicular delivery has been investigated as a potential means of getting drugs that affect the hair and pilosebaceous appendages to their sites of action. For example, Li *et al.* demonstrated that liposomally entrapped dyes and melanin can be delivered into the hair shafts of histocultured skin (Li et al. (1992) *In Vitro Cell Dev Biol* 28A:679-681; Li et al. (1993) *In Vitro Cell Dev Biol* 29A:192-194). Skalko *et al.* reported a greater efficiency of liposomal clindamycin over conventional formulations in the treatment of acne vulgaris (Skalko et al. (1992) *Int J Pharm* 85:97).

In a series of studies using a variety of polar and nonpolar permeants in different animal models, it has been demonstrated that the liposomes direct small and large molecules alike into follicles which, once there, can serve as a reservoir for protracted delivery into the dermis (Dowton et al. (1993) *STP Pharma Sciences* 3:404-407; Niemiec et al. (1994) *STP Pharma Sciences* 4:145-149; Hu et al. (1994) *STP Pharma Sciences* 4:466-469; Lieb et al. (1994) *Pharm Res* 11:1419-1423; Hu et al. (1996) *Drug Delivery* in press; Niemiec et al. (1995) *Pharm Res* 12:1184-1188; Fleisher et al. (1995) *Life Sci* 57:1293-1297). It is shown herein that substantial quantities of plasmid DNA can be delivered to the follicle via topical application of novel nonionic-cationic liposomal systems (see Examples 3-5).

Based on the invention described herein, it is possible to establish a hypothetical model explaining how nonionic liposomes faciliate delivery of macromolecules into skin. According to this hypothetical model, several factors play a role in facilitating nonionic liposome delivery of macromolecules into skin. These include: (i) liposomal dehydration as a driving force; (ii) penetration enhancing effects of components of the liposomal bilayers: (iii) drug/liposome interaction effects; and (iv) phase transition effects on the stratum corneum.

Preferred non-ionic components include a lipid vesicle having a primary wall material and a secondary wall material. The term "primary wall material" is used interchangeably herein with the term "primary lipid" and as used herein means that the lipid constitutes the greatest proportion (by weight) of any single lipid material forming the lipid bilayers or lipid vesicle, preferably 60% or greater. In a preferred embodiment,

the primary wall material is selected from the group consisting of C_{12}-C_{18} glyceryl mono- and diesters and C_{12}-C_{18} fatty alcohols. Preferred primary lipids are C_{16} and C_{18} fatty alcohols, glycol stearate, glyceryl monostearate, glyceryl distearate, and glyceryl dilaurate. Other primary lipids are also within the scope of the invention, so long as they form a non-ionic lipid vesicle with a secondary lipid together or not with other reagents. In a preferred embodiment of the invention, the primary lipid is glyceryl dilaurate.

The terms "secondary wall material" and "secondary lipid" are used intechangeably herein and is preferably selected from the group consisting of quaternary dimethyldiacyl amines (including ammonium derivatives such as chlorides), polyoxyethylene acyl alcohols (tradename BRIJ), polygycerols, fatty acids, and sorbitan fatty acid esters (tradename SPAN). Other secondary lipids are also within the scope of the invention, so long as they form a non-ionic lipid vesicle with a primary lipid together or not with other reagents. In a preferred embodiment, the secondary lipid is polyoxyethylene-10-stearyl ether.

In other preferred embodiment, the lipid vesicle further contains additional components or agents, such as charge producing agents. In a preferred embodiment, the lipid vesicle contains a sterol, such as cholesterol, a cholesterol salt or ester, phytocholesterol, or hyrdocortisone. Another preferred additive is α-tocopherol, preferably added at about 1% by weight of total lipids.

A particularly preferred non-ionic vesicle of the invention contains glyceryl dilaurate as the primary lipid, polyoxyethylene-10-stearyl ether as the secondary lipid, and cholesterol, preferably in a weight ratio of about 50:23:15, respectively.

These preferred non-phospholipid lipid vesicles are well known in the art and are described in patents such as United States Patent No. 4,911,928, United States Patent No. 5,032,457, United States Patent No. 5,147,723, and United States Patent No. 5,260,065, the disclosures which are incorporated herein by reference.

The choice of a cationic lipid for the preparation of a liposomal delivery system is dependent on its ability to transfect a wide variety of cells, the inherent toxicity of the lipid to the cells, and its compatibility with the non-ionic lipid vesicles. Accordingly, preferred cationic lipids include bilayer-forming cationic lipids containing unsaturated fatty acid chains. It has been shown previously that DOTMA, used alone, is capable of mediating transfection of specific cell types (Felgner *et al.* (1987) *Proc Natl Acad Sci USA* 84:7413-7417).

Accordingly, preferred cationic lipids are selected from the NC liposomal delivery system of the invention comprises a cationic lipid, which is preferentially

selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium bromide (DOTMA), 1,2-dimyristyloxypropyl-N,N-dimethyl-hydroxyethyl ammonium bromide (DMRIE), [N-(N', N'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), dioctadecylamidoglycyl spermidine (DOGS), dimethyl dioctadecylammonium bromide (DDAB), dioleoyl phosphatidylethanolamine (DOPE), 2,3-dioleoyloxyl-N-[2(sperminecarbozamide-0-ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1-[2-(oleoyloxy)-ethyl]-2-oleyl-3-(2-hydroxyethyl) imidazolinium chloride (DOTIM), 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP), 1,2-diacyl-3-trimethylammonium propane (TAP), 1,2-diacyl-3-dimethylammonium propane (DAP), and fatty acid salts of quaternary amines. TAP and DAP can have various chains (14:0, 16:0, 18:0, and 18:1) and various acyl groups (dimyristoyl, dipalmitoyl, distearoyl, and dioleoyl). Any cationic lipid that does not degrade the NC liposomal delivery system and is capable of delivering a nucleic acid into a cell, when used together with a non-ionic lipid vesicle can be used. The preferred cationic lipid for use in the invention is 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP). Cationic lipids are further described in the following references: Fasbender et al. (1995) *Am J Physiol* 269:L45-L51; Solodin et al. (1995) *Biochemistry* 34:13537-13544; Felgner et al. (1994) *J Biol Chem* 269:2550-2561; Stamatatos et al. (1988) *Biochemistry* 27:3917-3925; Leventis and Silvius (1990) *Biochim Biophys Acta* 1023:124-132.

A preferred NC liposomal delivery system of the invention contains glyceryl dilaurate as the primary lipid, polyoxyethylene-10-stearyl ether as the secondary lipid, cholesterol, and DOTAP as the cationic lipid, preferably in a weight ratio of about 50:23:15:12, respectively.

In one embodiment of the invention, the non-ionic lipid vesicle is prepared and subsequently mixed with the cationic lipid. Alternatively, the primary lipid and the secondary lipid of the non-ionic lipid vesicle are mixed together with the cationic lipid. Additional agents, e.g., lipids or chemical reagents, can be added at an appropriate time during the preparation of the NC liposomal delivery system. A preferred method for preparing the liposomal formulations of the invention consist of mixing appropriate amounts of the lipids and melting them together. Such a method is further described herein in the Examples.

Various combinations and ratios of primary wall material, secondary wall material, cationic lipid and additional agents (if any) can be used for preparing a liposome delivery system of the invention. The composition and ratio of the different components of the liposomal delivery system depends on the characteristics of each

component. The ratio may also vary according to the particular use of the liposomal delivery system and/or the contents of the liposomal delivery system. As set forth above, a preferred ratio between glyceryl dilaurate (GDL) as the primary lipid, cholesterol (CH), polyoxyethylene-10-stearyl ether (POE) as the secondary lipid, and 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP) as the cationic lipid is about 50:15:23:12 by weight, respectively. However, the amount and type of each component can be varied. For example, glyceryl distearate (GDS) can be used instead of GDL to form a liposomal formulation in a gel state that has been shown to facilitate retention of drugs in the stratum corneum via reservoir effects. Yet other preferred formulations include formulations in which the ratio of GDS: CH: POE: DOTAP are X: 15: Y: 12, wherein X is the weight percent of GDS and Y is the weight percent of POE and X ranges from about 45%-55% and Y ranges from about 18% to 28%. Another preferred formulation contains GDL:GDS:CH: POE: DOTAP in a ratio of 25:25:15:23:12 by weight. Also within the scope of the invention are such formulations in which the ratio of GDL:GDS is varied, such that the penetration enhancer effects of the GDL-based formulation are combined with the reservoir effects of the GDS-based formulation.

Liposomal suspensions are preferably sonicated, e.g., for about 20 minutes at room temperature, to reduce their size. Formulations having liposomes of uniform size (mean particle size of 100 ± 20 nm) can be obtained, as compared to formulations having liposomes of 500 ± 100 nm for the unsonicated NC liposomes. In the method of the invention, it is preferable to add the nucleic acid to the liposomes after sonication of the liposomes. The DNA/NC liposomal suspension is then preferably incubated at room temperature, e.g., for about 45 minutes, prior to topical administration. Formulations having a mean particle size greater than 2 μm can be obtained using such methods.

Preferred ratios of nucleic acid to lipid weight ratio are 1:1 and 1:2. However, ratios of about 1:3, 1:4, 1:5, 1:10, 1:25, 1:50, and intermediate ratios thereof, are also within the scope of the invention. Such ratios can vary according to various criteria, e.g., the type of constituents of the liposomal delivery system, the cell type to be transfected, and the type of nucleic acid.

Preferred sizes of the liposomes (not including the nucleic acid) in the NC liposomal formulations include liposomes of about 25 to about 500 nm, with 100 to about 250 nm preferred. The size of liposomes can be changed by various processes known to those skilled in the art. For example, the size of liposomes can be reduced by vortexing. Alternatively, the size of liposomes can be changed and/or rendered more uniform by extruding the liposomes through a series of straight-bore polycarbonate membranes of varying pore diameters at high pressure (250 psi) (Fleisher et al. *supra*).

A preferred ratio of nucleic acid to cationic lipid in the liposomal formulations of the invention is based on two factors: a) the maximum cationic lipid concentration that could be used without inducing toxic effects, and b) the amount of cationic lipid that would be required for near optimum complexation of the nucleic acid. A preferred ratio of nucleic acid to cationic lipid is about 0.4 μg of DNA per nmol of cationic lipid. For example, a lipid formulation can contain 12% weight DOTAP and a DNA concentration of about 7 mg/ml or about 0.4μg/nmol cationic lipid. However, ratios of about 0.25 to about 1.5 μg/nmol cationic lipid are also within the scope of the invention, where the DNA component is expressed by weight and the lipid component is expressed in molar quantity.

The size distribution of the liposomes and the liposome-DNA complexes, as well as their integrity and quality can be determined by various techniques. A Nikon Diaphot inverted light microscope can be used to visualize particles with diameters greater than 0.5μm. Alternatively, a NiComp 370 laser light scattering particle sizer can be used to visualize particles of smaller size.

It is preferred to use highly pure lipid components for the preparation of the liposome delivery systems of the invention. The use of highly purified components also reduces the possibility of *in vivo* toxicity associated with contaminants present in the lipids. Such highly purified lipids can be bought from manufacturers, such as ICI Americas or Sigma Chemicals.

Nucleic Acids and Transgenes

The nucleic acid introduced into cells, e.g., *in vivo*, can be any nucleic acid, including DNA, single stranded DNA, e.g., oligonucleotides, and RNA, e.g., mRNA, tRNA, and ribozymes. The nucleic acid can be small, containing a few nucleotides, or large, containing at least several kilobases. For example, the nucleic acid can be a yeast artificial chromosome (YAC). The nucleic acid can be a natural nucleic acid or prepared chemically. The term "nucleic acid", as used herein, is intended to include at least one nucleic acid molecule. Thus, a nucleic acid, as used herein, can be one, two, or more nucleic acid molecules. For example, a nucleic acid corresponding to two expression vectors can be mixed with the liposomal delivery system and introduced into a target cell, e.g., a follicular cell. The nucleic acid molecules can be linear or circular, depending on the type of nucleic acid.

A preferred nucleic acid is a DNA molecule with at least one expressible form of a transgene. The term "expressible form of a transgene" is intended to include a

transgene and all regulatory elements necessary for expression of the appropriate expression of the transgene. Such regulatory elements include basic promoters (e.g., TATA box sequence), enhancers, silencers, polyadenylation signals, and the like. These are further defined below.

The terms "regulatory element", "regulatory sequence", and "regulatory nucleic acid element" are used interchangeably herein and are intended to include any nucleic acid fragment having a specific nucleic acid sequence which, alone or together with one or more other nucleic acid fragments is capable of modifying the expression of a gene to which the regulatory sequence is operably linked. Modifying expression of a gene can be upregulating the expression of a gene or downregulating the expression of a gene. Regulatory elements are known to those skilled in the art and are described, e.g., in Goeddel (1990) Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA.

The term "promoter" is intended to include a nucleic acid which is capable of controlling the expression of a gene to which it is operably linked. A promoter typically contains at least one regulatory nucleic acid element. The term "5' flanking sequence" is intended to include a nucleic acid sequence located 5', i.e., upstream, of the transcription initiation site of a gene. The term 5" flanking sequence" is used interchangeably herein with the term "promoter".

The term "3' flanking sequence" is intended to include a nucleic acid sequence located 3', i.e., downstream of the polyadenylation signal of a gene.

"Operably linked" as applied to a transgene and regulatory element or promoter is intended to mean that the nucleotide sequence of a transgene, is linked to a regulatory sequence in a manner which allows expression of the transgene in a host cell. The term "operably linked" is intended to include any linkage that allows the regulatory sequence to control expression of the transgene of interest. In a preferred embodiment, a regulatory sequence has a chemical linkage to the gene of interest, such as natural linkage between two nucleic acid bases. The regulatory sequence or promoter can be linked directly to the transgene. Alternatively, the regulatory sequence can be linked indirectly to the transgene, such as by a linker. Such a linker can vary in length and may contain sequences that are recognized by restriction enzymes.

A regulatory element can be a portion of a promoter to which the RNA Polymerase binds and which contains a TATA box. Such a regulatory element is responsible for the basal or constitutive transcription of a gene and is classically referred to as a promoter. As used herein, however, the term "promoter" also refers to a larger fragment of DNA controlling gene expression.

A regulatory sequence can be an enhancer. Generally, an enhancer is an element which modifies the basal transcription of a gene which it controls. An enhancer element can be located several kilobases away from the gene whose expression it affects and it can be located in any part of a gene, such as in 3' or 5' non coding sequences and in introns. Regulatory elements also include silencers, i.e., DNA elements which will reduce the level of transcription of a gene.

Preferred promoters include strong promoters, such as viral promoters, e.g., a cytomegalovirus (CMV) promoter such as the CMV IE promoters. A preferred CMV promoter is the CMV early enhancer promoter, which is a powerful constitutive promoter. An even more preferred promoter is a truncated version of the CMV early enhancer promoter, such as the promoter included in the expression plasmid pCVC103. Other preferred viral promoters include SV40 promoters, e.g., the SV40 LTR promoter, the SV40 early promoter, and the SV40 later promoter. Other promoters include the AD2 MLP, the HSV TK, and the MMTV LTR.

Regulatory sequences within the scope of the invention include tissue specific regulatory sequences. The term "tissue specific regulatory element" is intended to include a regulatory element that stimulates expression of a gene to which it is operably linked predominantly in a specific tissue or cell type. A "tissue" as defined herein can be a an organ, e.g., kidney, liver, heart or a population of cells making up a structure, such as an epithelium. Tissue specific expression of a gene is also intended to include expression of the gene in a cell at a specific stage of differentiation and not in another stage of differentiation. Preferred tissue specific regulatory elements include regulatory elements that predominantly stimulate expression of a gene to which it is operably linked in a specific type of perifollicular cell. Accordingly, a preferred regulatory element stimulates gene expression predominantly in perifollicular fibroblasts. Other preferred regulatory elements stimulate gene expression predominantly in sebocytes. Accordingly, the invention provides methods for selectively expressing a gene in a particular type of perifollicular cell, even though the nucleic acid comprising the gene is delivered to all types of perifollicular cells.

Other preferred regulatory elements that can be used to control expression of the gene introduced into cells by the method of the invention include inducible regulatory elements. Such regulatory elements are responsive to specific agents, e.g., inducers. Accordingly, expression of an inducible element can be stimulated by an agent or repressed by an agent. Inducers within the scope of the invention include agents which interact with a receptor on the surface of a cell or within a cell, such as hormones, cytokines, and chemicals. Inducible regulatory systems for use in mammalian cells are

known in the art, for example systems in which gene expression is regulated by heavy metal ions (Mayo et al. (1982) *Cell* 29:99-108; Brinster et al. (1982) *Nature* 296:39-42; Searle et al. (1985) *Mol Cell Biol* 5:1480-1489), heat shock (Nouer et al. (1991) In: Heat Shock Response Nouer L. Ed., CRC, Boca Raton , FL, pp167-220), hormones (Lee et al. (1981) *Nature* 294:228-232; Hynes et al. (1981) *Proc Natl Acad Sci USA* 78:2038-2042; Klock et al. (1987) *Nature* 329:734-736; Israel & Kaufman (1989) *Nucl Acids Res* 17:2589-2604 and PCT Publication No. WO 93/23431), tetracycline (Gossen and Bujard (1992) *Proc Natl Acad Sci USA* 89:5547-5551 and PCT Publication No. WO 94/29442) or FK506 related molecules (PCT Publication No. WO94/18317). An inducer can also be light of a certain wavelength, such as ultraviolet light. Accordingly, the invention provides methods for delivering a nucleic acid to skin cells *in vivo* and methods for controlling expression of the genes delivered.

In view of the knowledge in the art relating to tissue specific and inducible regulatory elements, also within the scope of the invention are regulatory elements which have been modified at least in part. For example, a regulatory element that is tissue specific sebocytes can be engineered to become inducible by a specific compound by, e.g., operably linking the tissue specific regulatory element to a synthetic element responsive to the said compound.

The efficiency of a promoter or regulatory element to control expression of a gene tissue specifically can be determined by transfection of tissue culture cells or skin cells as described herein. Alternatively, methods of transfection other than by the liposomal delivery system of the invention can be used to determine efficiency of a promoter in tissue culture cells. For example, a promoter can be tested in tissue culture cells by transfection using calcium chloride methods, electroporation, or other methods known in the art.

The nucleic acid introduced into cells according to the method of the invention comprises at least one transgene. The term "transgene" is intended to include a gene which is introduced into a cell. The transgene can encode a protein or a peptide. Alternatively, the transgene can be a nucleic acid that is transcribed into RNA, but does not encode a peptide. For example, a transgene can be a nucleic acid which upon transcription into an RNA molecule is an "antisense" strand of another nucleic acid in the cell or outside the cell, such that upon expression of the transgene and synthesis of antisense molecules, a function in the cell is affected. In a more preferred embodiment of the invention the antisense nucleic acid inhibits or reduces expression of another nucleic acid, such as an endogenous nucleic acid.

The transgene can be any gene whose expression in perifollicular cells is beneficial to a specific condition. The transgene can also be a gene encoding a protein which is secreted and which is beneficial to cells surrounding the hair shaft, e.g., for treating a specific condition. Accordingly, the protein encoded by the transgene can be a secreted protein, e.g., a soluble protein, a membrane bound protein, or an intracellular protein. Preferred transgenes encode therapeutic proteins. A therapeutic protein can be a soluble protein, such as a ligand, a receptor, an agonist of a ligand, an agonist of a receptor, an antagonist of a ligand, and an antagonist of a receptor. The ligand can be, e.g., a hormone, a growth factor, or a cytokine.

A preferred soluble protein is an IL-1 receptor antagonist (IL-1ra). Interleukin-l receptor antagonist protein is a well characterized competitive inhibitor of IL-1 that is produced by several types of cells found within the skin, including monocytes, macrophages, fibroblasts, keratinocytes, and polymorphonuclear leukocytes (Bigler et al. (1992) *J Invest Dermatol* 98:38-44; Deleuran (1994) *Acta Derm Venereol Suppl* (Stockh) 189:1-34; Muzio et al. (1994) *Blood* 83:1738-1743). Several skin conditions, such as alopecia areata, psoriasis, atopic dermatitis and eczema, have been associated with aberrations in the expression of the proinflammatory cytokine IL-1 as well as IL-1 receptors and naturally occurring IL-l antagonists (Kondo et al. (1995) *J Invest Dermatol* 105:334-338; Hammerberg et al. (1992) *J Clin Invest* 90:571-583; Debets et al. (1995) *J Invest Dermatol* 105:480; Hoffmann et al. (1995) *J Invest Dermatol* 105:467; Wenzel et al. (1995) *J Invest Dermatol* 105:467). Furthermore, previous studies have suggested that appropriate levels of IL-lra expression in skin may inhibit the pathophysiological effects of IL-1 in human skin disease (Kondo et al. *supra*; Hammerberg et al. *supra*; Debets et al. *supra*; Hoffmann et al. *supra*; Wenzel et al. *supra*). Accordingly, the invention provides methods for treating skin diseases, e.g, skin diseases involving aberrations in the expression of IL-1, IL-1 receptor, or IL-1 antagonists. cDNA for human interleukin-l receptor antagonist protein is described in Arend et al. (1989) *J Immunol* 143:1851-1858, Arend et al. (1990) *J Clin Invest* 85:1694-1697, and Seckinger et al. (1987) *J Immunol* 139:1541-1545.

Another preferred soluble protein is a soluble or transdominant negative hormone receptor, such as an androgen receptor. Follicular cells transfected with a transdominant negative androgen receptor can have special applicability to the treatment of acne and male pattern baldness.

In another embodiment of the invention, the transgene encodes a suicide gene. A suicide gene is intended to include a gene which induce cell death, e.g., when the cell is exposed to a specific condition. Examples of suicide genes include genes encoding a

protein involved in apoptosis, such as Fas. Apoptosis in Fas-bearing cells is induced upon binding of a Fas ligand with the Fas receptor on the Fas-bearing cells. (Nagata and Golstein (1995) *Science* 267:1449). Other apoptotic genes include genes from the Bcl-2 family, such Bcl-Xs (Boise et al. (1993) *Cell* 74:597-608; Martin and Green (1995) *Critical Reviews in Oncology/Hematology* 18:137-153; and Savill (1994) *European Journal of Clinical Investigation* 24:715-723). Another suicide gene is the thymidine kinase (TK) gene. A cell expressing TK will be killed upon treatment with gancyclovir. In another embodiment of the invention, the suicide gene is a toxin, such as ricin. Accordingly, the method of the invention provides methods for eliminating or killing perifollicular cells. Such methods can be used, for example, to treat malignant or benign skin cancers or for cosmetic purposes.

In a specific embodiment of the invention, the transgene comprises a nucleotide sequence containing one or more open reading frames, i.e., sequences that code for peptides, such that upon transfection into perifollicular cells according to the method of the invention, at least one protein or peptide is synthesized in the perifollicular cell. Furthermore, the transgene can encode a single peptide or the transgene can encode several peptides.

In another embodiment of the invention, the transgene is a nucleotide sequence, which is expressed into one or more functional RNA molecules (e.g., an antisense RNA molecule or a ribozyme). In a preferred embodiment of the invention, the functional RNA molecule inhibits, or at least decreases, expression of one or more endogenous genes in the specific target cell. Thus, the method of the invention is useful for decreasing expression of a selected gene in perifollicular cells. For example, the level of a hormone, or hormone receptor can be reduced by introducing into perifollicular cells a nucleic acid encoding an RNA molecule which is antisense to the hormone or hormone receptor RNA according to the methods of the invention, such that translation of the hormone or hormone receptor RNA is inhibited or reduced.

An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid, e.g., complementary to an mRNA sequence encoding a protein, constructed according to the rules of Watson and Crick base pairing. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense sequence complementary to a sequence of an mRNA can be complementary to a sequence found in the coding region of the mRNA or can be complementary to a 5' or 3' untranslated region of the mRNA. Preferably, an antisense nucleic acid is complementary to a region preceding or spanning the initiation codon or in the 3' untranslated region of an mRNA. For a discussion of the regulation of gene expression

using antisense genes see Weintraub et al. (1986) "Antisense RNA as a molecular tool for genetic analysis", *Reviews - Trends in Genetics*, Vol. 1(1).

In another embodiment of the invention, the transgene encodes a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. A ribozyme having specificity for a nucleic acid of interest can be designed based upon the nucleotide sequence of the nucleic acid. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in an mRNA of interest. See for example Cech et al. U.S. Patent No. 4,987,071; and Cech et al. U.S. Patent No. 5,116,742.

Alternatively, an antisense nucleic acid or ribozyme can be synthesized *in vitro* and introduced into perifollicular cells according to the method of the invention. Methods for *in vitro* production of antisense nucleic acids and ribozymes are known in the art.

For introducing an expressible form of a transgene into perifollicular cells using a liposomal delivery system of the invention, it is preferable that the transgene is contained in a plasmid or vector containing sequences or elements well known in the art for preparing the nucleic acid prior to transfection. Such sequences include those that enable the nucleic acid to be replicated, such as a bacterial origin of replication. Suitable plasmid expression vectors include CDM8 (Seed (1987) *Nature* 329:840) and pMT2PC (Kaufman et al.(1987) *EMBO J* 6:187-195). Other preferred elements which can be included in the expression vector or plasmid include elements allowing episomal maintenance and/or autonomous replication of the plasmid containing the transgene (see below) or elements favouring chromosomal integration of the transgene.

In one embodiment of the invention, the expressible form of a transgene introduced into perifollicular cells is in a viral vector. Viral vectors include recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1. Nucleic acids introduced into cells using these vectors become stably integrated into the chromosomal DNA of the host cell. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag, pol, env*) is replaced by a gene of interest rendering the retrovirus replication defective.

Other viral vectors which can be used include adenovirus-derived vectors (See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155). Introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis *in situ*ations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Expression of the transgene of interest can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral expression vector system useful for expression of a transgene is the adeno-associated virus (AAV). Adeno-associated viruses exhibit a high frequency of stable integration (see for example Flotte et al. (1992) *Am J Respir Cell Mol Biol* 7:349-356; Samulski et al. (1989) *J Virol* 63:3822-3828; and McLaughlin et al. (1989) *J Virol* 62:1963-1973). A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc Natl Acad Sci USA* 81:6466-6470; Tratschin et al. (1985) *Mol Cel. Biol* 4:2072-2081; Wondisford et al. (1988) *Mol Endocrinol* 2:32-39; Tratschin et al. (1984) *J Virol* 51:611-619; and Flotte et al. (1993) *J Biol Chem* 268:3781-3790). Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses.

It is preferable to use purified nucleic acids for use in the liposomal delivery system of the invention. For example, in view of the lipophilic properties of liposomes, contamination of plasmid DNA with bacterial lipids and or polysaccharides is preferably avoided. Such contaminants can also contribute to the potential toxicity of the liposomal formulations. Therefore, it is preferable to use highly purified plasmid DNA, e.g., pharmaceutical grade manufactured under Food and Drug Administration standards of Good Laboratory Practices, (GLP) for the method of the invention.

It is further preferable to use a nucleic acid having an appropriate physical conformation. The physical state of plasmid DNA (supercoiled, closed circular, nicked, linear) can effect the amount of DNA per unit volume, the amount of DNA that can associate in an appropriate physicochemical orientation with charged liposomes of a defined size, and probably can effect the 'bioavailability' of the plasmid (partially degraded plasmid is unavailable for transcription). Accordingly, it is preferable to use nucleic acids which are supercoiled in the liposomal delivery system of the invention.

Supercoiling of DNA is controlled by the regulated activity of topoisomerases. Topoisomerase II (DNA gyrase) in an ATP dependent process enzymatically converts DNA into negatively supercoiled double helices. Purified DNA gyrase can be used as a biochemical reagent for induction of supercoiling in plasmid DNA (Bates and Maxwell (1989) *EMBO J* 8:1861-1866; Cozzarelli (1980) *Science* 207:953-960; Kreuzer and Cozzarelli (1980) *Cell* 20:245-254; Marini et al. (1980) *J Biol Chem* 255:4976-4979; Maxwell and Gellert (1986) *Adv Protein Chem* 38:69-107). Thus, in one embodiment, the expression plasmid DNA is treated with a DNA gyrase prior to final FPLC (fast performance liquid chromatography) or equivalent chromatographic purification in order to achieve a uniform physical structure within the plasmid DNA. For example, plasmid DNA can be incubated with DNA gyrase (Gibco BRL) in the presence of 1 mM ATP in order to induce formation of negative supercoils. Accordingly, a preferred plasmid or vector containing the transgene has been gyrase treated and chromatographically sized purified.

Final composition of the liposomal formulations are preferably performed in a laminar flow environment using sterile techniques immediately before use of the reagents *in vivo*.

Molecules, such as macromolecules, other than nucleic acids, can also be introduced into cells according to the method of the invention. For example, the liposomal delivery system can be used to deliver a proteinous compound. A proteinous compound is intended to include any compound which has at least one amino acid. Preferred proteinous compounds include compounds which are hydrophilic and anionic compounds.

Pharmaceutical compositions

The liposomal formulations of the invention and the nucleic acid can be administered topically to a skin having hair follicles. Accordingly, in a preferred embodiment, the liposomal formulations are in a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent

is incompatible with the liposomal formulation, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The liposomal formulations and the nucleic acid are topically applied to the subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the liposomal formulations in which any toxic effects are outweighed by the therapeutic effects of the agent. The term "subject" is intended to include living organisms in which an immune response can be elicited, e.g., mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. Administration of a therapeutically active amount of a nucleic acid/liposomal formulation of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a nucleic acid/liposomal formulation, may vary according to factors such as the condition to be treated, disease state, the density of hair follicles on the skin, the type of skin, and the ability of the liposomal formulation to elicit a desired response in the subject. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

Under ordinary conditions of storage and use, the liposomal preparations may contain a preservative to prevent the growth of microorganisms or other agent preventing degradation of liposomes.

<u>Uses for NC liposomal formulations</u>

The ability to deliver expression plasmid DNA or other nucleic acid to follicular skin cells via topical application of a liposomal formulation allows the treatment of many dermatologic diseases. Several categories of dermatologic diseases could be treated according to the method of the invention: diseases of a follicular nature, infectious diseases, neoplastic diseases, autoimmune diseases, and acquired conditions. For all of these diseases, the general strategy is similar; transfection of accessible cells in order to mediate the expression of a transgenic protein having biological effects proximal to the site of application. In the case of transgenic secreted proteins, the biological effects can extend regionally beyond the immediate microenvironment of the transduced cells. In one embodiment, the invention provides a method for the

modulation of cell adhesion molecules and pro-inflammatory cytokines via the expression of soluble receptors or receptor antagonist proteins, such as for the treatment of autoimmune skin diseases including psoriasis and alopecia areata. In another embodiment, the expression of soluble or transdominant negative androgen receptors by transfected follicular cells can be used for treating acne and male pattern baldness.

The method of the invention further provides methods for treating skin diseases, such as alopecia areata, psoriasis, atopic dermatitis and eczema. Since these conditions have been associated with aberrations in the expression of the proinflammatory cytokine IL-1 as well as IL-1 receptors and naturally occurring IL-1 antagonists (Kondo et al. *supra*; Hammerberg et al. *supra*; Debets et al. *supra*; Hoffmann et al. *supra*; Wenzel et al. *supra*), the invention provides methods for modulating the amount of these proteins according to the disease, such that the skin disease is improved or cured.

Also within the scope of the invention are liposomal gene delivery systems which are administered systemically. Accordingly, the invention provides methods for treating diseases for which systemic administration of a nucleic acid is preferable.

This invention is further illustrated by the following Exemples which should not be construed as limiting. The contents of all references and published patent applications cited throughout this application are hereby incorporated by reference.

Examples

The following methodology was used in the Examples.

Preparation and Purification of Plasmid DNA

Expression plasmid used in these studies were derived from the eukaryotic expression vector pSG5 (Stratagene), and utilizes the SV40 LTR as a promoter and the SV40 polyadenylation sequence. The cDNA for human interleukin-1 receptor antagonist protein was cloned into the BamHI site of pSG5 to yield pSG5IL-1ra. The gene for *E. coli* β-galactosidase was cloned into the BamHI site of pSG5 to yield pSG5lacZ. The orientation of the transgene within recombinant plasmids was confirmed using a combination of restriction endonuclease mapping and dideoxynucleotide sequencing. Plasmid DNA was prepared from the DH5-α strain of *Escherichia coli* transformed with the recombinant plasmids and grown in LB broth containing ampicillin (50μg/ml). Plasmid DNA was purified using a modification of the alkaline lysis method followed by two separate bandings in cesium chloride equilibrium gradients (Sambrook

et al. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Ethidium bromide was extracted from the DNA with butanol and the batches were then dialyzed extensively against purified water (Millipore) and ethanol precipitated. Aliquots of the plasmids were then resuspended in purified water, filter sterilized through 0.22 micron filters (Millipore) and stored at -20°C until use. The purity and concentration of all the plasmid preparations were confirmed by electrophoresis in 1% agarose gels containing ethidium bromide, and UV spectroscopy at 260 nm and 280 nm (Beckman Spectrometer). DNA was also screened for the presence of bacterial endotoxin (Sigma). Batches with an endotoxin concentration exceeding 15 endotoxin units per ml were discarded. In the case of pSG5IL-1ra, all batches of purified DNA were tested for the presence of recombinant human IL-1ra protein by ELISA. None of the DNA used for the *in vivo* studies contained detectable levels of human IL-1ra (detection limits less than 29 pg/ml). Fluorescently labeled pSG5IL-1ra plasmid was produced in an identical fashion except that ethidium bromide intercalated into the DNA during the purification process was retained and excess non-incorporated ethidium bromide was removed by extensive dialysis in Tris-EDTA buffer.

Preparation of Nonionic/Cationic-based Liposomal Formulations

The nonionic/cationic (NC) liposomal formulations used in the experiments contained glyceryl dilaurate (GDL; Sigma Chemicals, >90% 1,3, isomer), cholesterol (CH; Sigma Chemicals), polyoxyethylene-10-stearyl ether (POE-10; ICI Americas), and 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP; Avanti Polar Lipids) at a weight percent ratio of 50:15:23:12. The lipid mixture also contained α-tocopherol (1% by weight of total lipids; Eastman Kodak). Appropriate amounts of the lipids were mixed and melted at 70°C in a sterile polystyrene centrifuge tube. The lipid melt was then filtered through a 0.22 μm filter (Nuclepore®) and the filtrate was reheated in a water-bath at 70°C prior to being drawn into a sterile syringe. A second syringe containing sterile, autoclaved, double-distilled water was preheated to 65°C and connected *via* a 3-way sterile stopcock to the lipid phase syringe. The aqueous phase was then slowly injected into the lipid phase syringe. The mixture was rapidly passed back and forth between the two syringes while being cooled under cold tap water until the mixture was at room temperature and stored at 4°C until use. The total lipid concentration in the suspension was 100 mg/ml.

The resulting NC liposomal suspensions were examined using a Nikon Diaphot light microscope to assure integrity and quality of the liposomal preparations.

Immediately before use in the *in vivo* experiments, the liposomal suspension was sonicated for 20 minutes at room temperature and an equal volume of aqueous pSG5IL-1ra or pSG5lacZ DNA solution (7 mg DNA/ml) was added by inversion mixing followed by incubation at room temperature for 45 minutes. Control formulations contained no DNA.

Preparation of Phospholipid-based Liposomal Formulations

Large unilamellar liposomes (LUV) were prepared containing egg phosphatidylcholine (PC; Avanti Polar Lipids)/CH /DOTAP and α-tocopherol (1% by weight of total lipids; Eastman Kodak) in a 1:0.5:0.1 molar ratio. A reverse-phase evaporation method based on that reported by Szoka and Papahadjopoulos (Szoka F., Jr. and Papahadjopoulos D. (1978) *PNAS USA* 75:4194-4198) was used in the preparation of the LUVs. The lipids were dissolved in a chloroform-methanol mixture (2:1 [v/v]). Isotonic 0.05 M HEPES buffer (Sigma Chemical Co.), pH 7.4, was added to form a clear mixture. The solvent-to-buffer ratio was 6:1 (v/v). The solvents were removed using a rotoevaporator (Buchli) maintained at 45°C. The total lipid concentration in the suspension was 75 mg/ml. The resulting liposomal suspensions were examined using a Nikon Diaphot light microscope to assure integrity and quality of the liposomal preparations. The liposomes were then sonicated for 20 minutes at room temperature before the addition of an equal volume of aqueous pSG5IL-1ra DNA solution (7 mg/ml). The liposomal DNA mixture was then allowed to incubate at room temperature for 45 minutes prior to topical application to the ventral side of hamster ears.

In vivo Experiments

Animal experiments were conducted under institutional guidelines with approval by the University of Michigan Committee on the Use and Care of Animals (UCUCA). Male golden Syrian hamsters, 10 weeks old, were purchased from Charles River Breeding Laboratories (Wilmington, MA) and were maintained for two weeks at a photoperiod of 14 hours (hr) of light and 10 hr of darkness to maximize androgen dependent sebaceous gland activity and thus control their size (Hoffmann K. (1979) *Prog Brain Res* 52:397-415). The ventral side of the hamster ears were carefully shaved one day prior to the experiments. The hamsters were anesthetized with a single dose of sodium pentobarbital (0.25 ml of 40 mg/kg i.p. injection). Following anesthetization, 50 μl of the test formulation containing the pSG5IL-1ra plasmid DNA were applied to the ventral surface of one ear, twice daily for three days. The contralateral ear was treated with an equivalent amount of liposomes without plasmid DNA (control). Additionally,

a set of control animals (n=4) were treated as described above with NC liposomes containing pSG5lacZ plasmid DNA. The total amount of lipid applied per ear of NC based liposomes was 15 mg (2.5 mg/dose), and the total lipid applied per ear with the PC-based liposomes was 11.25 mg (1.875 mg/dose). For both NC and PC based formulations the total amount of DNA applied was 1.05 mg (0.175 mg/dose). Fifteen hours after the last application of the test formulations, the hamsters were sacrificed and the ears excised by dissection across the base. Kinetics of transgene expression following topical application of NC liposomal pSG5IL-1ra plasmid DNA and blank NC liposomes was studied by sacrificing treated animals at 1, 3, 5 and 8 days after the last application. For each given time point, at least three animals were used per formulation tested. Ears of untreated animals were also used as negative controls. All experiments were carried out under non-occluded conditions. At the time of sacrifice the ears were isolated by sharp dissection, weighed and measured along each border (in order to calculate the surface area exposed to treatment), then processed as described below. One ml of blood was also collected from each animal immediately following sacrifice and centrifuged at 14,000 rpm (Beckman) for 10 minutes at 4°C. The serum was collected and assayed for IL-1ra content.

Confocal Laser Scanning Microscopy

Ear tissue was embedded in O.C.T. (Miles Laboratories, Elkhart Lake, IN) and frozen in isopentane containing dry ice. The tissue blocks were stored at -80°C before sectioning. Blocks were sectioned at 20 µm using a Tissue-Tec II Model 4553 cryostat (Miles Laboratories, Inc., Naperville, IL) and placed on poly-L-lysine double-coated slides. Immediately before viewing, the slides were mounted with Vecta-Shield mounting media (Vector Laboratories, Burlingame, CA). The sections were examined using a Bio-Rad MRC 600 laser scanning confocal microscope (Bio-Rad Microscience Div., Cambridge, MA) and analyzed with CoMOS program. The imaging procedure employed has been described in detail earlier (Paddock et al. (1991) *Biotechniques* 11:486-493). The light source was argon-krypton laser equipped with 520 nm filter wheel and cubes for detection of emitted light. Images were resized using Adobe PhotoshopTM Version 3.0 (Adobe Systems, Inc., Mountain View, CA). Prints of the digital images were made using a Kodak XLS 8600 PS printer (Eastman Kodak Co., Rochester, NY).

Southern Analysis

Animals treated with the NC liposomal pSG5IL-1ra plasmid DNA formulations (50 mg/ml NC liposomes, 3.5 mg/ml DNA) were sacrificed at 1, 4, 6, 8, 12 and 24 hours after application. Those treated with the aqueous DNA solutions (3.5 mg/ml), were examined at 12 and 24 hours after application. Following sacrifice, the ears were cut at the base and pinned to a board with the ventral side facing up. The ventral side was swabbed several times with PBS soaked tissue and stripped with tape until all hair was removed and the surface looked shiny indicating complete removal of the stratum corneum. The surface of the ventral ear was swabbed several times again with PBS soaked tissue before the ventral ear was separated from the underlying cartilage. The ventral ear was placed on a 60 mm polystyrene dish with the epidermal side facing down. 500 μl of a 0.05 M Isotonic HEPES, pH 7.4 was placed on the ear and the sebaceous glands were carefully scraped using a sterile, dull scalpel. This procedure was carried out twice. Tissues were then minced in 1 ml of homogenate buffer and collected in sterile Eppendorf tubes along with the gland scrapings. Genomic DNA was isolated from the tissue homogenates using DNAzolTM reagent (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer's protocol. The concentration of DNA isolated from each sample was determined spectrophotometrically.

Samples (10 μg of total DNA) were loaded onto 0.8% agarose gels, electrophoresed at 20 V for 21 hours, and transferred to a Nytran membrane. Controls included lanes containing known amounts of pSG5IL-1ra (15 or 150 μg), both undigested and linearized by digestion with EcoR1. The membrane was probed with [^{32}P]-labeled oligonucleotide probes specific for human IL-1ra generated from a 650 bp fragment of human IL-1 ra cDNA that was isolated from pSG5IL-1ra using random hexanucleotide primers, [^{32}P]-dCTP (Amersham), and Klenow fragment (BRL). Hybridization buffer included 10% dextran sulfate, 35% formamide, 5X SSC, 0.1% SDS and 100 μg/ml salmon sperm DNA, and were performed overnight at 42°C. Membranes were washed twice for 10 min at room temperature in 2X SSC/0.1% SDS, twice for 10 minutes at 65°C in 1XSSC/0.1% SDS, and once for 10 minutes at 65°C in 0.1XSSC/0.1% SDS. Membranes were wrapped in plastic and exposed to film (Kodak X-AR5) at -70°C using intensifying screens.

Detection of IL-1ra by Immunohistochemistry

Treated ears were embedded in OCT medium (Miles, Inc., Eikhart, IN) and frozen in isopropyl alcohol containing dry ice. The frozen samples were stored at -80°C

before sectioning. Serial sections (5 µm) were obtained using a cryostat (Tissue-Tee II Model 4553, Lab-Tec Products, Miles Laboratories, Inc., Naperville, IL) and placed on poly-L-lysine double-coated slides. The tissue sections were then immediately fixed in 100% acetone for 10 minutes at 4°C and processed using a Histostain-SP AEC kit (Zymed Lab, Inc., South San Francisco, CA) according to the manufacturer's recommendations. The sections were reacted with primary murine anti-human IL-1ra (29 ng/ml, Bachem Bioscience, Inc., King of Prussia, PA) for 30 minutes. After completion of the protocol, slides were counterstained with hematoxylin, rinsed a final time and mounted with 100 µl of GVA-mount before being examined and photographed using a Nikon Optiphot microscope (Nikon, Tokyo, Japan).

Isolation of Soluble Proteins from Various Strata of the Hamster Ear

Each ear strata (ventral skin, auricular cartilage and dorsal skin) was isolated from the ear using sharp dissection as follows. Following sacrifice, the ears were cut at the base and pinned to a board with the ventral side facing up. The ventral side was swabbed several times with PBS soaked tissue and stripped with tape until all hair was removed and the surface looked shiny indicating complete removal of the stratum corneum. The surface of the ventral ear was then swabbed several times again with PBS soaked tissue before the ventral ear was separated from the underlying cartilage. The ventral ear was then placed on a sialinized glass slide with the epidermal side facing down. 500 µl of a homogenate buffer (0.05 M Isotonic HEPES, pH 7.4, 0.1% PMSF, 0.1% HSA) was placed on the ear and the sebaceous glands were carefully scraped using a sterile, dull scalpel. This procedure was carried out twice. The ventral skin strata was then minced in 1 ml of homogenate buffer and collected in sterile Eppendorf tubes along with the gland scrapings. The cartilage and dorsal skin were minced in 1 ml of homogenate buffer each. The samples were then sonicated on ice for 2 minutes and centrifuged at 15,000 rpm for 10 minutes at 4°C. The supernatants were collected immediately and analyzed for human IL-1ra protein.

Detection of IL-lra Protein

Human IL-1ra protein was measured using a human specific IL-1ra protein immunoassay (Research and Diagnostics Systems, Minneapolis, MN). Tissue homogenates were assayed in triplicate and the optical density of each sample was determined using a spectrophotometer set to 450 nm with the correction wavelength set to 570 nm. A standard curve to 2000 pg/ml was also prepared using the homogenate buffer as the diluent and negative control. The detection limit of this ELISA was 29

pg/ml. The test samples were compared to the standard curve to determine IL-1ra concentration. The results were expressed as 10^{-9} gm human IL-1ra/cm^2 and/or 10^{-9} gm human IL-1ra/gm tissue.

Example 1: **<u>Hybrid non-ionic/cationic (NC) liposome-DNA complexes mediate gene transfer *in vitro*</u>**

This Example compares the efficiency of non-ionic lipids, cationic lipids, e.g., LIPOFECTIN™, and combinations thereof to mediate transfection of DNA into cells in culture. Five liposomal formulations, listed in Table I, and plasmid DNA alone were used for *in vitro* transfections.

The DNA used in these transfections was the eukaryotic expression plasmid DNA (pSG5lacZ), containing the gene for *E. coli* β-galactosidase. Successful transfectants were detected by histochemical staining with the chromogenic substrate X-gal.

Table I - Composition of hybrid non-ionic/cationic liposomal formulations

Formulation	Lipids	pSG5lacZ
1	6 μg of LIPOFECTIN™ cationic liposomes	4 μg
2	50 μg non-ionic liposomes*	4 μg
3	50 μg non-ionic liposomes + 6 μg LIPOFECTIN™	4 μg
4	44 μg non-ionic liposomes + 6 μg DOTAP#	4 μg
5	44 μg non-ionic liposomes + 6 μg DODAP+	4 μg
6		4 μg

* Non-ionic liposomes = glyceryl dilaurate:cholesterol:polyoxyethylene-10-stearyl ether (57:15:28)

DOTAP = 1,2-dioleoylox-3-(trimerhylammonio) propane

+ DODAP = 1,2-dioleoyloxy-3-(dimethylammonio) propane

All of the novel liposomal formulations were prepared immediately before use and were tested in parallel (in triplicate) for the ability to mediate transfection of plasmid DNA (pSGSlacZ) into 293 cell monolayers. The transfections were performed by combining the liposomal-DNA formulations (total volume = 100 μl) with 1 ml of serum-free, antibiotic-free Dulbecco's Modified Eagle Medium. This mixture was overlaid gently onto the 293 cells in 24 well plates and incubated for 5 hours at 37°C. Cells were returned to complete media for 48 hours after transfection, then fixed and reacted with X-gal solution (X-gal solution is 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside in PBS).

The number of transfectants resulting from each formulation was determined by visual examination of monolayer cultures for the presence of blue staining (lacZ+) cells using inverted light microscopy. The relative transfection efficiency observed for each formulation was determined as the ratio of blue cells per test formulation versus the number of blue cells observed using the Lipofectin formulation (positive control). These results were converted to a linear scale and recorded in Table II.

The results presented in Table II indicate that, as expected, LIPOFECTIN™/DNA (positive control) was able to mediate transfection of the 293 cells, and DNA alone (negative control) was unable to mediate transfection. Non-ionic liposomes combined with LIPOFECTIN™ did not inhibit the relative efficiency of transfection and resulted in a formulation that even enhances the ability of the cationic liposomes to mediate

transfection. The addition of the cationic lipid DODAP to the standard non-ionic formulation did not result in successful transfection. The formulation of nonionic lipids supplemented with DOTAP yielded the highest number of transfectants per microgram of DNA used. Thus, NC formulations containing 12 weight % DOTAP and DNA concentration of approximately 8 mg/ml , corresponding to a DNA to DOTAP ratio of 0.4 μg/nmol, gave the best transfection efficiency.

Table II - Hybrid non-ionic/cationic liposomal formulations can mediate transfection of DNA *in vitro*.

Formulation	Relative transfection efficiency
1. (LIPOFECTIN™)	+
2. (GDL/CH/POE)	0
3. (GDL/CH/POE + LIPOFECTIN™)	++
4. (GDL/CH/POE/DOTAP)	+++
5. (GDL/CH/POE/DODAP)	0
6. DNA	0

GDL = glyceryl dilaurate, CH = cholesterol, POE = polyoxyerhylene-10-stearyl ether, DOTAP = 1,2-dioleoyloxy-3-(trimethylammonio) propane. DODAP = 1,2-dioleoyloxy-3-(dimethylammonio) propane.

These results indicate that formulations consisting of mixtures of nonionic/cationic liposomes or the addition of particular cationic lipids (to facilitate transfection) to nonionic liposomes (to facilitate targeting) can effectively deliver plasmid DNA in an expressible form into cells *in vitro*.

Example 2: <u>Sonication of liposomes prior to the addition of DNA allows delivery of intact DNA to cells</u>

This Example shows how different methods for the preparation of a topical liposome-DNA formulation can affect the integrity of the expression plasmid DNA. Liposome-DNA formulations were initially prepared by simple mixing followed by

sonication according to previously described methods (Li et al. (1995) *supra*). Examination of the resulting formulations by electrophoresis in agarose gels revealed that sonication of the liposome-DNA mixture results in physical fragmentation of the plasmid DNA. To avoid this, a method that employed sonication of the liposomes prior to addition of the plasmid DNA was developed. This method yielded formulations that were more uniform in size (mean particle size = 100 ± 20 nm) compared to a mean particle size of 500 ± 100 nm for the unsonicated NC liposomes. Addition of plasmid DNA to the sonicated liposomes did not reveal evidence of fragmentation or degradation of the plasmid DNA as determined by electrophoresis of the topical formulations in agarose gels. The particle size of the plasmid DNA-NC liposome formulation had a mean particle size greater than 2 μm.

Example 3: <u>NC liposomal formulations efficiently deliver plasmid DNA into the hair follicles</u>

This Example shows the ability of these novel topical NC formulations to deliver plasmid DNA into cells *in vivo*, as shown by histologic localization of plasmid DNA in hamster skin following topical application of nonionic-cationic (NC) liposomes containing DNA.

The ventral surface of the hamster ear was treated with a single dose of NC liposome containing pSG5IL-1ra DNA (0.175 mg) that had been fluorescently labeled with ethidium bromide. Controls were treated with fluorescently labeled pSG5IL-1ra DNA alone (0.175 mg). At 6 and 24 hours after application of the formulation, the animals were sacrificed and the ears were isolated then snap frozen in OCT. Serial cryosections (20 μm) were placed onto glass slides, mounted with Vecta-Shield mounting media (Vector Laboratories, Burlingame, CA). The sections were examined using a Bio-Rad MRO 600 laser scanning confocal microscope (Bio-Rad Microscience Div., Cambridge, MA), using an argon-krypton laser and analyzed with CoMOS program.

Plasmid DNA was visualized along the epidermal surface and within the perifollicular regions of the skin 6 hours after topical application of a single dose of the NC liposomal formulation. Although some of the plasmid DNA continued to be visualized on the skin surface 6 hours after application, the delivery of the labeled DNA into the hair follicles and perifollicular glands appeared to be complete by 24 hours post administration. Control animals treated with an aqueous formulation containing an

equivalent dose of the fluorescently labeled plasmid failed to show evidence of DNA beyond the superficial epidermis after 24 hours of observation. This indicates that perifollicular delivery is a physicochemical property specific to NC liposomal formulations. Furthermore, since the intensity of ethidium bromide fluorescence is directly proportional to the amount of double stranded DNA present in the formulation, these qualitative results indicate that some of the plasmid DNA was delivered to the region of the pilosebaceous unit in the form of non-degraded plasmid (LePecq and Paoletti (1967) *J Mol Biol* 27:87-106).

Example 4: <u>NC liposomal formulations deliver intact DNA to *in vivo* tissues</u>

This Example shows that the plasmid DNA observed *in vivo* in the follicle following topical application of a NC-plasmid complex to skin, as described in Example 3, is mostly intact plasmid DNA.

The ventral surface of the hamster ear was treated with a single dose of NC liposome containing pSG5IL-1ra DNA (0.175 mg), or liposome alone. Duplicate animals treated with NC liposomes + DNA were sacrificed at 6, 12 and 24 hours after treatment. A pair of animals treated with the NC liposomes alone were examined 24 hours after topical treatment. Following sacrifice, the ears were isolated, depilated, minced, and total genomic DNA was isolated from the tissue homogenates. Ten µg of total genomic DNA was electrophoresed in a 0.8% agarose gel, then transferred to a Nytran membrane. Controls included a lanes containing 15 and 150 pg of purified pSG5IL-1ra (p_{15}, p_{150} respectively), and duplicate amounts of pSG5IL-1ra linearized by digestion with EcoR1. The membrane was probed with [^{32}P]-labeled oligonucleotide probes specific for human IL-1ra, washed and exposed to film (Kodak X-AR5) at -70°C using intensifying screens.

The Southern blot hybridization, a photograph of which is shown in Figure 1, shows the presence of bands specific for pSG5IL-1r in the NC liposome treated skin beginning 6 hours after administration. Qualitatively, the amount of pSG5IL-1ra present within the skin remained constant from 12 to 24 hours after treatment. Although some of the plasmid (p) DNA was identified in the form of supercoiled (sc) plasmid at 6 hours post-treatment, from 12 to 24 hours post-treatment most of the plasmid DNA was in the form of closed circular (cc) or linearized plasmid (linear). No bands were observed in the genomic DNA obtained from animals treated with liposomes alone.

Thus, the results indicate that most of the plasmid DNA present within the skin was in the form of closed circular or linearized plasmid. The results further show that for the first 24 hours post-administration, plasmid DNA was not subjected to progressive degradation and suggests that some of the plasmid was delivered intracellularly and may have been protected from digestion by extracellular nucleases.

Example 5: **<u>NC liposomal formulations function as transfecting agents *in vivo*</u>**

The previous Examples showed that NC formulations deliver intact plasmid DNA to *in vivo* tissues. This Example demonstrates that the NC liposomal formulations mediate transfection of cells of the tissue which are proximal to the *in vivo* location of the delivered expression plasmid DNA.

The ventral surface of the hamster ear was treated twice daily for three consecutive days with NC liposomes containing plasmid DNA. Controls were treated with an equivalent dose of NC liposomes without DNA. 24 hours after application of the last dose the ears were isolated and snap frozen in OCT. Serial cryosections (5 μm) were then obtained from the treated ears and placed onto glass slides. Sections were fixed in ice cold acetone blocked and reacted with monoclonal antibodies against human IL-1ra (murine IgG1, 29 ng/ml) or irrelevant murine IgG1 (29 ng/ml). Sections were counterstained with hematoxylin. Sections were examined by light microscopy (Nikon UFX-DX) for the presence of IL-1ra expressing cells (red staining cells) and representative photomicrographs were obtained.

The results of the *in situ* immunohistochemical staining show that the NC liposomal formulation also functioned as a transfecting agent. Transfected human IL-1ra expressing cells were identified within the follicles in the proximal third of the hair shaft and at the base of the hair shaft. Negative controls treated with aqueous formulations of expression plasmid DNA, or liposomes alone failed to show evidence for IL-1ra expressing perifollicular cells.

Thus, NC liposomal formulations efficiently mediated transfection of cells *in vivo*.

Example 6: **Kinetics of expression of DNA transfected with NC liposomal formulations**

This Example shows the kinetics of hIL-1ra expression within treated skin over an 8 day period following a multiple dose (twice daily for three days) topical application protocol.

Formulations were applied to the ventral surface of hamster ears twice daily for 3 days (total dose of pSG5IL-1ra DNA was 1 mg). The concentration of human IL-1ra present in whole tissue homogenates obtained from different strata on days 1, 3, 5, and 8 post treatment was determined using ELISA. Assays were performed in triplicate and results expressed as pg hIL-1ra surface area of treated skin.

The results are presented in Figure 2. Transgenic expression of human IL-1ra in the skin of the ventral ear was detected at its highest levels 15 hours after application of the final topical dose. The levels of transgene expression remained significantly above control values ($p<0.005$) on days 1 to 5, and had returned progressively to baseline levels by day 8. Ear cartilage and dorsal skin were also assayed for transgenic human IL-1ra, however all of these values were at or below the detection limits of the ELISA (29 pg/ml) and no significant differences were observed between animals treated with NC liposomal DNA and those treated with NC liposomes alone. It was also found that samples of ventral ear, glands, cartilage and dorsal skin obtained from control animals treated with NC liposomes + pSG5lacZ plasmid DNA exhibited transgenic human IL-1ra levels that were below the detection limits of the assay. In addition, no transgenic human IL-1ra was detected in the serum of the treated or control animals. These results indicate that expression of transgenic protein is confined to tissues locally targeted by the NC liposomal pSG5IL-1ra plasmid DNA formulation, and that the diffusion of transgenic IL-1ra protein is largely confined to the microenvironment proximal to the point of topical application. These results corroborate immunohistochemical analysis of treated skin showing expression of transgenic hIL-1ra *in vivo*.

Example 7: **NC liposome formulations are more efficient transfection agents than phospholipidic/cationic liposome formulations**

The dynamics of plasmid mediated hIL-1ra expression obtained *in vivo* from the NC liposome formulation were then compared with those associated with a conventional

PC liposome carrier system. Accordingly, this Example compares expression of transgenic human IL-1ra in the ventral ear of the hamster following topical *in vivo* application of nonionic/cationic (NC) and phospholipid/cationic (PC) liposome formulations with and without pSG5IL-1ra plasmid DNA.

Formulations were applied to the ventral surface of the ear twice daily for 3 days (total dose of DNA was 1 mg). 24 hours after application of the last topical dose the concentration of transgenic human IL-1ra present in whole tissue homogenates obtained from the ventral ear was determined using ELISA. Assays were performed in triplicate and results are expressed as pg IL-1ra/cm^2 of treated skin surface area.

Although the charged lipid-DNA ratios were different for the two formulations, the total dose of DNA applied to the hamster ears was identical for the two systems. Under these conditions, it was found that the NC+DNA formulation mediated levels of transgene expression that were approximately four times higher than those obtained with the PC+DNA formulation ($p<0.001$) (Figure 3). In fact, ears treated with the NC liposomes + DNA expressed a mean of 165.5 ± 15.6 pg/cm^2 human IL-1ra, those treated NC liposomes alone expressed a mean of 31.4 ± 24.0 pg/cm^2 human IL-1ra. Ears treated with PC liposomes + DNA expressed a mean of 40.8 ± 8.1 pg/cm^2 human IL-1ra and those treated with PC liposomes expressed mean levels of human IL-1ra below the limits of detection by ELISA (< 29 pg/cm2). Controls treated with DNA alone expressed a mean of 32.5 ± 11.0 pg/cm^2 of human IL-1ra. Levels of human IL-1ra protein detected in animals treated with PC liposome formulations were not significantly different from the levels detected in animals treated with PC liposomes alone ($p=0.991$) or NC liposomes alone ($p=0.915$).

Thus, NC liposome formulations are more efficient in mediating *in vivo* transfection of cells than PC liposome formulations.

The foregoing examples illustrate the effectiveness of the present invention. These examples are merely exemplary and those skilled in the art will be able to determine other modifications to the described procedures which fall within the scope of the invention. Accordingly, the invention is defined by the following claims and equivalents thereof.

In the Claims

1. A method of delivering a nucleic acid to a mammalian cell *in vivo* comprising the steps of;

providing a liposomal delivery system for said nucleic acid, said liposomal delivery sytem comprising a lipid vesicle having a primary wall material and a secondary wall material, said primary wall material being selected from the group consisting of C_{12}-C_{18} glyceryl mono- and diesters and C_{12}-C_{18} fatty alcohols and said secondary wall material being selected from the group consisting of quaternary dimethyldiacyl amines, polyoxyethylene acyl alcohols, polyglycerol fatty acids, and sorbitan fatty acid esters; said liposomal delivery system further containing a cationic lipid;

incorporating said nucleic acid into said liposomal delivery system to produce a nucleic acid/liposomal delivery system; and

topically administering said nucleic acid/liposomal delivery sytem to said mammalian cell at a location of the skin having hair follicles.

2. The method of claim 1, wherein said mammalian cell is associated with follicular units.

3. The method of claim 2, wherein said mammalian cell is in a pilosebaceous unit.

4. The method of claim 2, wherein said cell is a perifollicular cell.

5. The method of claim 2, wherein said cell is a keratinocyte.

6. The method of claim 1, wherein said cationic lipid is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium bromide (DOTMA), 1,2-dimyristyloxypropyl-N,N-dimethyl-hydroxyethyl ammonium bromide (DMRIE), [N-(N', N'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), dioctadecylamidoglycyl spermidine (DOGS), dimethyl dioctadecylammonium bromide (DDAB), dioleoyl phosphatidylethanolamine

(DOPE), 2,3-dioleoyloxyl-N-[2(sperminecarbozamide-0-ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1-[2-(oleoyloxy)-ethyl]-2-oleyl-3-(2-hydroxyethyl) imidazolinium chloride (DOTIM), 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP), 1,2-diacyl-3-trimethylammonium propane (TAP), 1,2-diacyl-3-dimethylammonium propane (DAP), and fatty acid salts of quaternary amines.

7. The method of claim 6, wherein said cationic lipid is 1,2-dideoyloxy-3-(trimethylammonio) propane (DOTAP).

8. The method of claim 6, wherein said primary wall material is selected from the group consisting of glyceryl dilaurate and glyceryl distearate.

9. The method of claim 7, wherein said second wall material is polyoxyethylene-10-stearyl ether.

10. The method of claim 1, wherein said nucleic acid comprises an expressible form of a transgene.

11. The method of claim 10, wherein said transgene encodes a protein.

12. The method of claim 11, wherein said protein is selected from the group consisting of a ligand, a receptor, an agonist of a ligand, an agonist of a receptor, an antagonist of a ligand, and an antagonist of a receptor.

13. The method of claim 12, wherein said protein is a soluble protein.

14. The method of claim 13, wherein said soluble protein is an IL-1 receptor antagonist.

15. The method of claim 13, wherein said soluble protein is a transdominant negative hormone receptor.

16. The method of claim 10, wherein said transgene expresses antisense RNA.

17. The method of claim 1, wherein said mammalian cell is in a subject having a disorder and the method is for treating said disorder.

18. The method of claim 17, wherein said disorder is a dermatological disorder.

19. The method of claim 18, wherein said dermatological disorder is selected from the group consisting of infectious diseases, neoplastic diseases, autoimmune disorders, alopecia, aquired conditions, acne, baldness, atopic dermatitis, and eczema.

20. The method of claim 19, wherein said autoimmune disorder is psoriasis or alopecia areata.

21. A nucleic acid/liposomal delivery system for delivering a nucleic acid topically to a mammal *in vivo* comprising:

a nucleic acid and a liposomal delivery system for said nucleic acid, said liposomal delivery sytem comprising a lipid vesicle having a primary wall material and a secondary wall material, said primary wall material being selected from the group consisting of C_{12}-C_{18} glyceryl mono- and diesters and C_{12}-C_{18} fatty alcohols and said secondary wall material being selected from the group consisting of quaternary dimethyldiacyl amines, polyoxyethylene acyl alcohols, polyglycerol fatty acids, and sorbitan fatty acid esters; and wherein said liposomal delivery system further contains a cationic lipid.

22. The nucleic acid/liposomal delivery system of claim 22, wherein said cationic lipid is selected from the group consisting of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium bromide (DOTMA), 1,2-dimyristyloxypropyl-N,N-dimethyl-hydroxyethyl ammonium bromide (DMRIE), [N-(N', N'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), dioctadecylamidoglycyl spermidine (DOGS), dimethyl dioctadecylammonium bromide (DDAB), dioleoyl phosphatidylethanolamine (DOPE), 2,3-dioleoyloxyl-N-[2(sperminecarbozamide-0-ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 1-[2-(oleoyloxy)-ethyl]-2-oleyl-3-(2-hydroxyethyl) imidazolinium chloride (DOTIM), 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP), 1,2-diacyl-3-trimethylammonium

propane (TAP), 1,2-diacyl-3-dimethylammonium propane (DAP), and fatty acid salts of quaternary amines.

23. The nucleic acid/liposomal delivery system of claim 22, wherein said cationic lipid is 1,2-dideoyloxy-3-(trimethylammonio) propane (DOTAP).

24. The nucleic acid/liposomal delivery system of claim 23, wherein said primary wall material is selected from the group consisting of glyceryl dilaurate and glyceryl distearate.

25. The nucleic acid/liposomal delivery system of claim 24, wherein said second wall material is polyoxyethylene-10-stearyl ether.

26. The nucleic acid/liposomal delivery system of claim 21, wherein said nucleic acid comprises an expressible form of a transgene.

27. The nucleic acid/liposomal delivery system of claim 26, wherein said transgene encodes a protein.

28. The nucleic acid/liposomal delivery system of claim 27, wherein said protein is selected from the group consisting of a ligand, a receptor, an agonist of a ligand, an agonist of a receptor, an antagonist of a ligand, and an antagonist of a receptor.

29. The nucleic acid/liposomal delivery system of claim 28, wherein said protein is a soluble protein.

30. The nucleic acid/liposomal delivery system of claim 29, wherein said soluble protein is an IL-1 receptor antagonist.

31. The nucleic acid/liposomal delivery system of claim 27, wherein said transgene expresses antisense RNA.

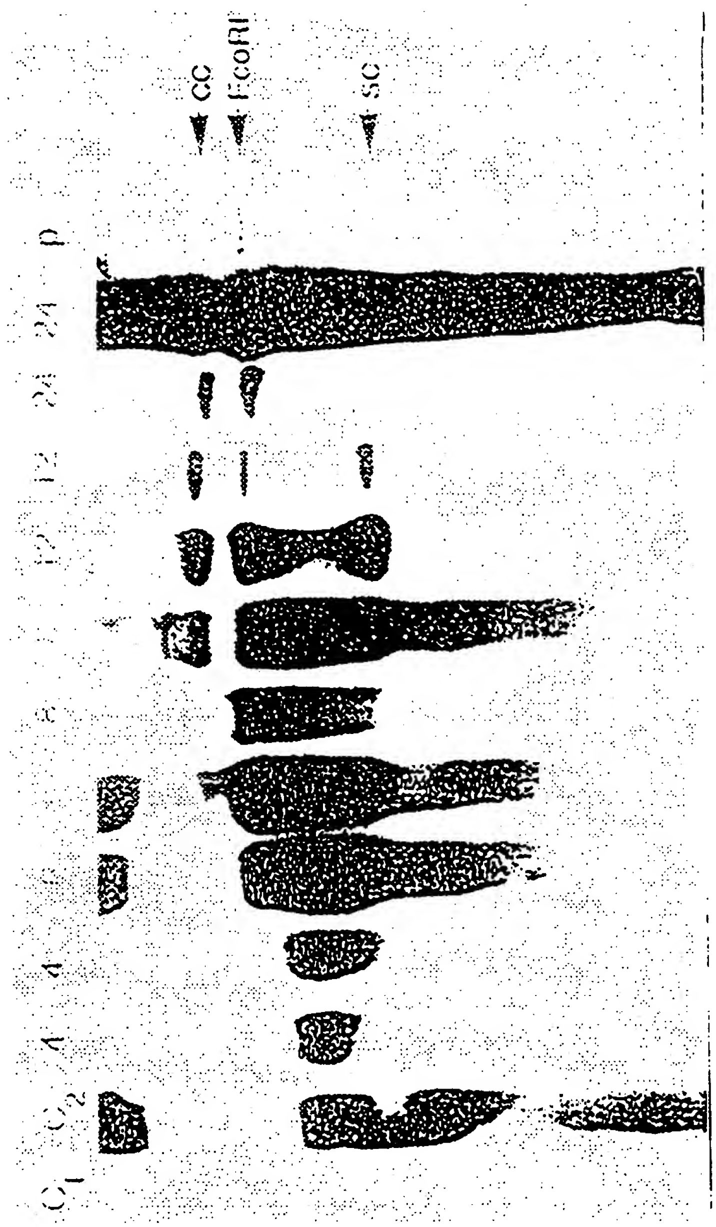

FIG. 1

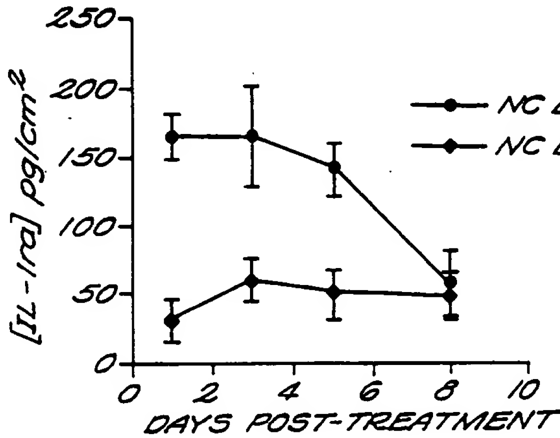

FIG. 2A

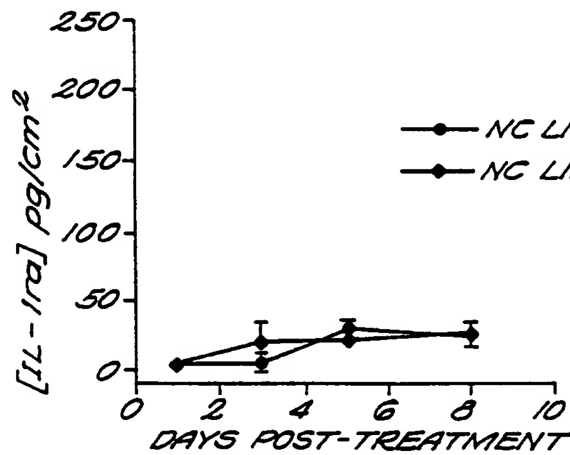

FIG. 2B

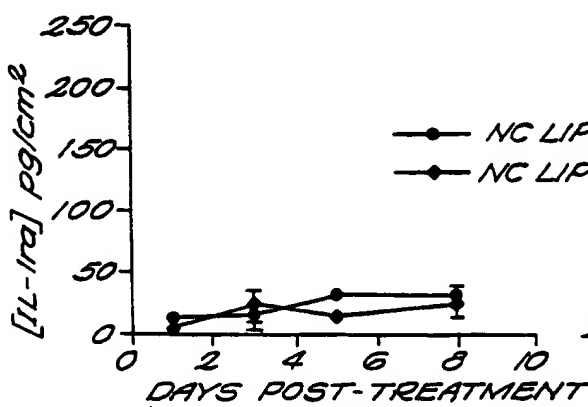

FIG. 2C

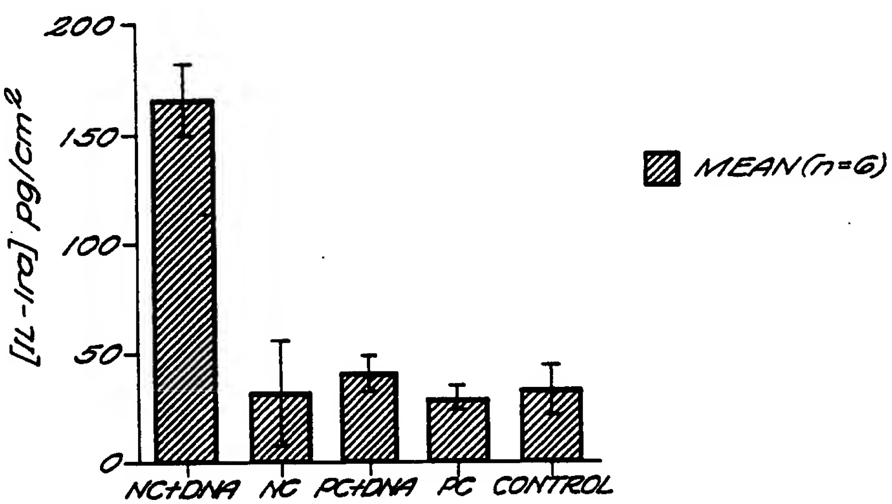

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07645

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 9/127, 48/00; C12N 15/09, 15/11, 15/79, 15/88
US CL :424/450; 435/69.1, 320.1, 172.3; 514/44; 536/23.1, 23.5, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450; 435/69.1, 320.1, 172.3; 514/44; 536/23.1, 23.5, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JAMES W. Towards gene-inhibition therapy: a review of progress and prospects in the field of antiviral antisense nucleic acids and ribozymes. Antiviral Chemistry & Chemotherapy. 1991, Vol. 2, No. 4, pages 191-214, especially page 198.	1-31
A	ORKIN et al. Report and Recommendations of the Panel to Assess the NIH Investment in Research on Gene Therapy. U.S. National Institutes of Health, Bethesda, Maryland. 07 December 1995, 41 pages, see especially pages 1-2.	1-31
A, P	VERMA et al. Gene therapy - promises, problems, and prospects. Nature. 18 September 1997, Vol. 389, pages 239-242, especially page 239, column 1.	1-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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Date of the actual completion of the international search	Date of mailing of the international search report
14 MAY 1998	30 JUL 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Deborah Freese /for SCOTT D. PRIEBE Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/07645

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GEWIRTZ et al. Facilitating oligonucleotide delivery: helping antisense deliver on its promise. Proceedings of the National Academy of Science USA. April 1996, Vol. 93, pages 3161-3163.	1-31
A	HAMMERBERG et al. Interleukin-1 receptor antagonist in normal and psoriatic epidermis. Journal of Clinical Investigation. August 1992, Vol. 90, pages 571-583, especially page 571 and pages 581-582.	1-31

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International application No.
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B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS
search terms: liposome#, non(-)ionic, lipid(w)vesicle#, DNA, nucleic, transgen##, RNA, deoxyribonucleic, ribonucleic, glyceryl, glycerol, distearate, monostearate, polyoxyethylene, ether, alcohol, stearyl, DAP, TAP, DOSPA, DOTIM, DDAB, DOTMA, DMRIE, DOGS, DOPE